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DISTRIBUTION AND METABOLISM OF α-ECDYSONE IN PUPAE OF THE SILKWORM *ANTHERAEA POLYPHEMUS*[1]

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The insect steroid α-ecdysone is known to provoke the adult development of diapausing insect pupae or even of isolated segments of pupae (Williams, 1970). Within 12–24 hours after the injection of 5–10 μg of the hormone into a diapausing saturniid pupa, the animal's respiration increases dramatically, and striking increases in the rates of synthesis of DNA, RNA, and protein can be demonstrated (Wyatt, 1968). These metabolic events precede by 3 or 4 days the first gross morphological change, the retraction of the epidermis from the overlying pupal cuticle.

Attempts to understand ecdysone's action at the molecular level are complicated by striking gaps in present knowledge. For example, ecdysone's target tissues have not yet been identified. Nor do we know whether it is unaltered α-ecdysone or a derivative which reacts with these tissues. The object of the present investigation was to clarify some of these matters by studying the fate of injected α-ecdysone, in particular, its chemical transformations and interactions with the tissues. Recent studies of estradiol in mammalian systems (Jensen and Jacobson, 1962; Gorski, Toft, Shyamala, Smith and Notides, 1968) have demonstrated the potential power of this approach. Some experiments of this kind on larval *Calliphora* have been reported by Karlson, Sekeris and Mauer (1964).

In the present communication we describe the distribution and metabolism of α-ecdysone in polyphemus pupae. Future publications will discuss the binding of the hormone in greater detail.

Materials and Methods

Experimental animals

Diapausing pupae of the silkworm, *Antheraea polyphemus*, were purchased from dealers and stored at 5° C until immediately before use. The pupae used in these experiments were chilled 5–10 months; though some began visible development within a day after removal from the cold, none exhibited retraction of the epidermis at the beginning of any experiment. Male pupae were used exclusively, since their large antennae were a convenience in some experiments.

Reagents

β-ecdysone was purchased from Mann Research. Other ecdysones were supplied as follows: synthetic α-ecdysone, synthetic α-ecdysone-23,24-^{3}H (7.3

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c/mmole), 25-deoxyecdysone-^{3}H, by Dr. John Siddall of Zoecon Corporation; ponasterone A, ponasterone C, by Professor K. Nakanishi of Columbia University; rubrosterone, by Professor T. Takemoto of Tohoku University. The radiochemical purity of the α-ecdysone-^{3}H was greater than 99% as determined by thin layer chromatography. Inulin-^{3}H (200 mc/g) was purchased from Schwarz BioResearch; water-^{3}H, from New England Nuclear Corporation.

Injections

Injections were made through the thoracic tergum using a 26-gauge hypodermic needle on a plastic 1 ml syringe controlled by an Agla micrometer. Each syringe was calibrated before use. Injections were routinely made without anesthesia, the puncture wound being promptly sealed with melted wax. Ecdysones were dissolved in 10% 2-propanol and injected in volumes not exceeding 50 μl; inulin-^{3}H and water-^{3}H were injected in no more than 50 μl of water.

Blood samples

Blood samples, except where otherwise indicated, were squeezed from a small incision in the integument at the edge of an antenna. When successive samples were taken from the same pupa, the incision was opened for each sample, and then resealed with melted wax.

Washing of antennae

Each antennae was removed, intact, separated from the overlying cuticle, and placed immediately into a beaker containing 50 ml of pupal saline (Weevers, 1966, modified by the substitution of sucrose for glucose and by the addition of 0.01 *M* thiourea and 0.1% penicillin) at room temperature. By means of a 23-gauge hypodermic needle, about 7 ml of this solution was injected through the main longitudinal blood space of the antenna; the injection rate was just sufficient to keep the antenna distended. The beaker was then placed on a low-speed gyratory shaker for 15 min, the solution being changed twice.

Extraction of α-ecdysone and its metabolites

Two extraction procedures were used: (A.) 0.2–0.5 ml of frozen blood was thawed and added to 2 ml of cold methanol. The resulting suspension was centrifuged at 12,000 $\times$ *g* for 10 min. The pellet was washed with 1 ml of cold methanol and again centrifuged. The supernatants were combined and the solvent evaporated under nitrogen at room temperature to approximately the original volume of the blood; the methanolic extract was stored at $-20°$ C until used. Any precipitate that formed during storage was discarded.

(B.) 10 μl of blood was removed from a pupa and added to 50 μl of methanol at room temperature. (Occasionally, 25 μl of blood and 100 μl of methanol were used.) The mixture was shaken vigorously, stored at $-20°$ overnight, and then centrifuged. The methanolic supernatant was used for chromatography. This method permitted sequential analyses of the blood of a single pupa.

Thin layer chromatography

Plastic-backed thin layer plates of silica gel containing fluorescent indicator (Eastman "Chromagram") were stored over calcium sulfate as desiccant. 10 μl samples were spotted after the addition of α-ecdysone and β-ecdysone (4 μg each) as markers. The solvent system was that of Horn, Middleton and Wunderlich (1966) (4 parts chloroform, 1 part 96% ethanol). The developed chromatogram was examined under an ultraviolet lamp, and the positions of the marker ecdysones were observed as dark spots. The chromatogram was then cut into strips 1–1½ cm wide, each containing the path of one sample. Each strip was cut into sections 2 mm long in the region containing α- and β-ecdysones, and 5 mm long elsewhere. The radioactivity in each section was measured in a liquid scintillation counter as described below. The pattern of radioactivity was analyzed graphically, using cpm/cm as the ordinate, and the position of the center of the section as abscissa.

Scintillation counting

Radioactivity was measured in a liquid scintillation counter, using 10 ml of a scintillation fluid consisting of 1 part Bio Solv type 3 (Beckman Instruments) to 10 parts toluene counting solution (0.4% 2,5-diphenyloxazole and 0.005% 1,4,bis-2-(methyl-5-phenyloxazolyl)-benzene in toluene). Tissue samples were placed in a scintillation vial containing 0.1 ml of a solution of sodium ethylenediaminetetraacetate (15 m*M*) and sodium dodecyl sulfate (1%), and warmed about 10 minutes on a hotplate before the counting solution was added. We were able to count whole antennae in this manner without significant quenching. Blood samples and sections of thin layer plates were added directly to the scintillation fluid. The customary 10 μl blood samples gave no quenching; the sections of thin layer plates gave substantial, but reproducible, quenching.

Results

The distribution of injected inulin and water

We studied first the rates at which two model compounds, inulin and water, are distributed after being injected into the hemocoel. We also determined the equilibrium distribution volume of each compound, *i.e.*, the volume through which each compound would have to be distributed to give the observed concentration, if the distribution were even.

Radioactive inulin was injected into a series of pupae *via* the right half of the thoracic tergum, and 10 μl blood samples were collected repeatedly from the left forewing and antenna of each animal. In 50% of the pupae, the inulin concentration reached equilibrium within two hours. Figure 1a shows a typical result. Figure 1b is included to demonstrate that before equilibrium is reached the concentrations at two separate sites sometimes differ dramatically. The inulin volume remained constant ($\pm$ 4%) for 24 hours after equilibrium was attained.

From the specific activity of the blood samples at equilibrium, we calculated the distribution volume. Figure 2 shows the equilibrium distribution volume for inulin as a function of live weight. The inulin volume is approximately 0.49 ml per

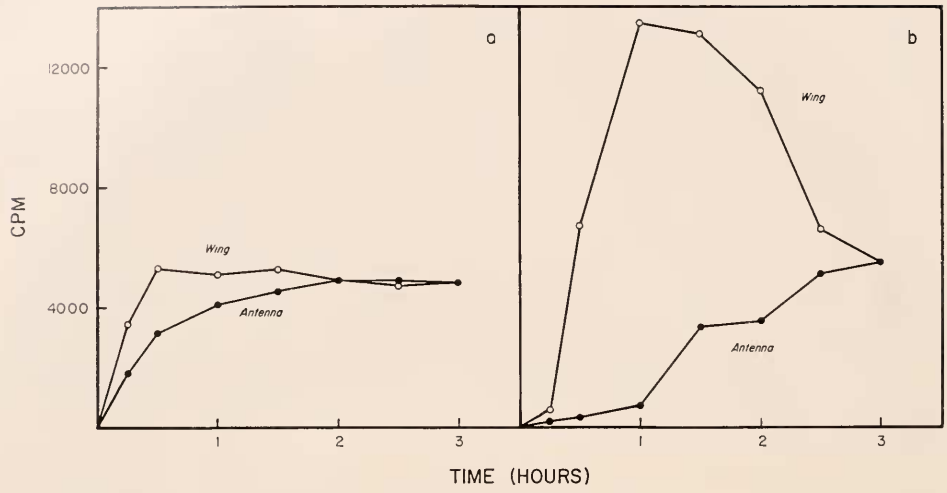

FIGURE 1. Distribution of inulin by the blood. Parts a and b represent different pupae; each was injected with 50 μl of inulin-^{3}H (7×10^5 cpm) via the thoracic tergum. The animals were kept at 25° C and 10 μl blood samples were taken at the times indicated. Abscissa: cpm per 10 μl of blood; open circles: blood samples taken from the wing; closed circles: blood samples taken from the antenna.

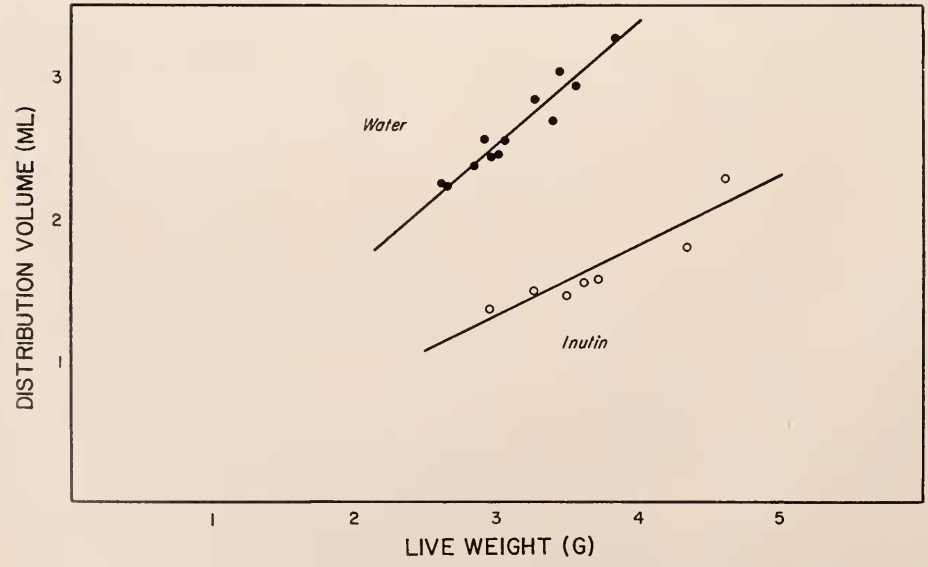

FIGURE 2. Distribution volume of inulin and water as a function of live weight. Each pupa received 50 μl (3×10^5 cpm) of inulin-^{3}H in water or 50 μl of water-^{3}H (6×10^6 cpm) injected *via* the thoracic tergum. After 3 hours (inulin) or 25 hours (water) at 25° C, a 10 μl aliquot of blood was removed from the antennal region and counted. The lines were calculated by the least-squares method.

g live weight. For reasons to be discussed later, we will assume that inulin is confined to the blood.

The water volume was measured in the same way and is approximately 0.86 ml per g live weight (Figure 2). The time required for water-^{3}H to reach its equilibrium concentration was not measurably different from that required for the equilibration of inulin. The water volume remained constant for at least 24 hours after equilibrium was attained.

The distribution of injected ecdysone

Radioactive α-ecdysone, injected in doses ranging from 1.0 to 8.6 μg per pupa, approached its equilibrium concentration in the blood of the antenna at a rate indistinguishable from that for inulin. This situation was not altered when 100 μg of unlabeled β-ecdysone and 1.2 μg of radioactive α-ecdysone were injected together: the radioactive material still reached its equilibrium distribution volume in about two hours.

In another experiment a large number of pupae were injected with 0.1 to 10 μg of α-ecdysone-^{3}H, and the blood of each pupa was sampled once during the 48 hours following the injection. Figure 3 illustrates this experiment for four different doses of ecdysone. Here the ordinate, the fraction of the radioactivity remaining in the blood, is simply the estimated inulin volume divided by the observed ecdysone volume. It is to be emphasized that the term "ecdysone volume" refers in fact to the distribution volume of radioactive α-ecdysone and its *radioactive* metabolites. There is a plateau between 3 and 36 hours, during which time the ecdysone volume is essentially constant and equal to 1.03 ± 0.13 (S. D.) ml per g live weight. This figure implies that 47% of the radioactivity is present in the blood. Only in the case of the 10.4 μg dose, which is sufficient to provoke adult development, are there pronounced deviations from the "equilibrium" level of 47%.

The ecdysone concentration in fat body and Malpighian tubules

Two pupae were injected with 1 μg and 10 μg doses of α-ecdysone-^{3}H (10^7 dpm in each case). One day later the pupae were dissected. Most of the Malpighian tubules were removed from each pupa, rinsed in saline, and tested for radioactivity. The cellular volume of this tissue is negligible; the radioactivity did not exceed background. Two other pupae were injected with 1 μg and 10 μg of α-ecdysone, respectively. Twenty-nine hours later 25–50 μl of fat body, including a small amount of blood, was removed with a pipette and counted. Each sample contained approximately the amount of radioactivity found in an equal volume of blood from the same pupa. Hence we conclude that the ecdysone that disappeared from the blood was not concentrated in the fat body or Malpighian tubules in the form of α-ecdysone or any radioactive metabolite of α-ecdysone.

The ecdysone concentration in the epidermis

Measurement of the radioactivity in an antenna was complicated by the large volume of blood trapped within this hollow organ. In order to measure the radioactivity associated with the cells of an unwashed antenna, we performed the

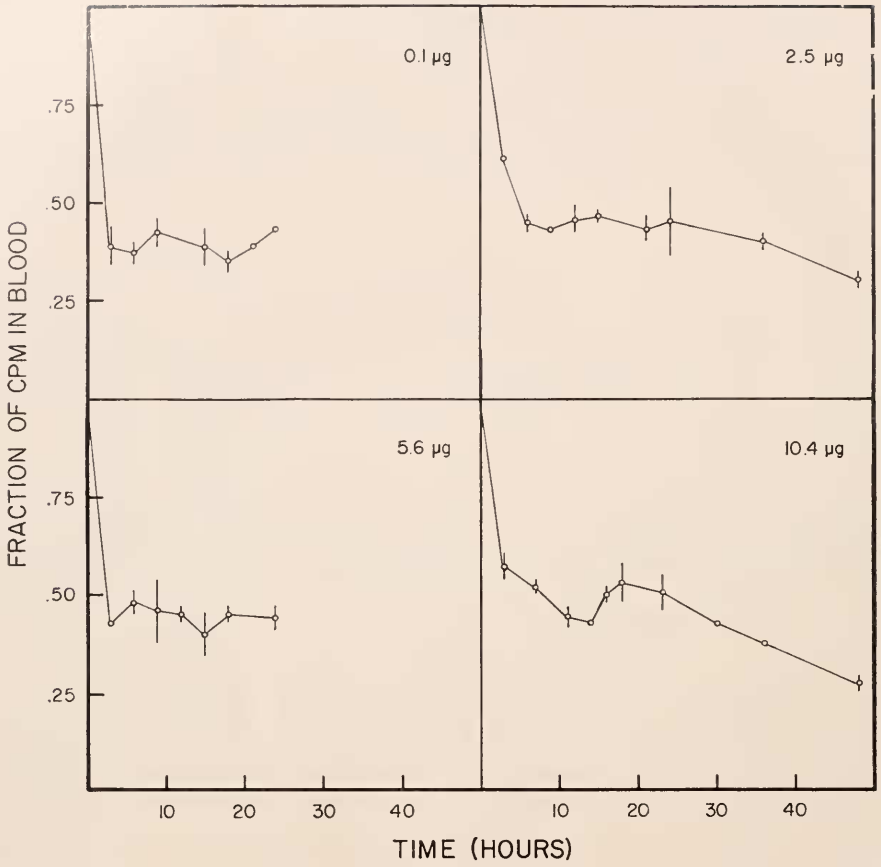

FIGURE 3. Fraction of radioactivity remaining in the blood after an injection of α-ecdysone-^{3}H at time zero. Each point is an average of values from two pupae; the range of individual values is indicated. In calculating the fractions, it was assumed that the blood volume is equal to the inulin volume shown in Figure 2.

following experiment. One series of pupae received inulin-^{3}H, and a second received α-ecdysone-^{3}H (2.5 µg/pupa). The antennae were removed from each pupa seven hours later; a blood sample was collected at the same time. Each antenna was blotted carefully and its volume measured by drawing it into a calibrated glass capillary. Assuming inulin to be present only in the blood, we found that about 70% of the volume of a blotted antenna was blood. This information enabled us to calculate the specific activity of the cells from pupae that had received α-ecdysone. We found no difference between the specific activity of the cells and that of the blood. Only differences greater than 30% would have been detected by this method.

Another group of pupae were injected with 0.1 to 10 µg of α-ecdysone-^{3}H. After 3–49 hours, we removed and washed their antennae as described under Methods, and counted the radioactivity. We had determined, using inulin, that

an antenna washed in this manner retains about 0.2 μl of blood. Therefore, blood samples were also counted and the radioactivity in each antenna was corrected for contamination with 0.2 μl of blood. In most cases, each antenna contained an amount of radioactivity equivalent to 1.4 ± 0.7 (S. D.) μl of blood. (Some important exceptions to this result are described in the Discussion.) Despite the uncertainties in the volume measurements, this experiment and the preceding one demonstrate that ecdysone and its radioactive metabolites in antennal tissue are largely unaffected by this washing procedure.

The metabolic products of α-ecdysone

Each of ten pupae was injected with 1.2 μg of α-ecdysone-^{3}H. At intervals from 3 to 24 hours later, pupae were exsanguinated and the blood extracted according to Method A. The final methanol fractions contained 82 (± 4)% of the radioactivity in the blood samples. There was no variation of extraction efficiency with time between injection and extraction. A control blood sample, to which α-ecdysone-^{3}H was added after bleeding, was also extracted with 82% efficiency. As is shown below, α-ecdysone is converted into a variety of metabolites during this 24-hour period; the invariance of the extraction efficiency gave assurance that the extraction procedure did not exclude any significant, radioactively-labeled metabolite of α-ecdysone, *i.e.*, any metabolite containing more than 5% of the radioactivity. More reproducible kinetic data on the appearance of metabolites were obtained using sequential samples from a single pupa, extracted according to Method B. Here again, no systematic variation in the efficiency of extraction was noted.

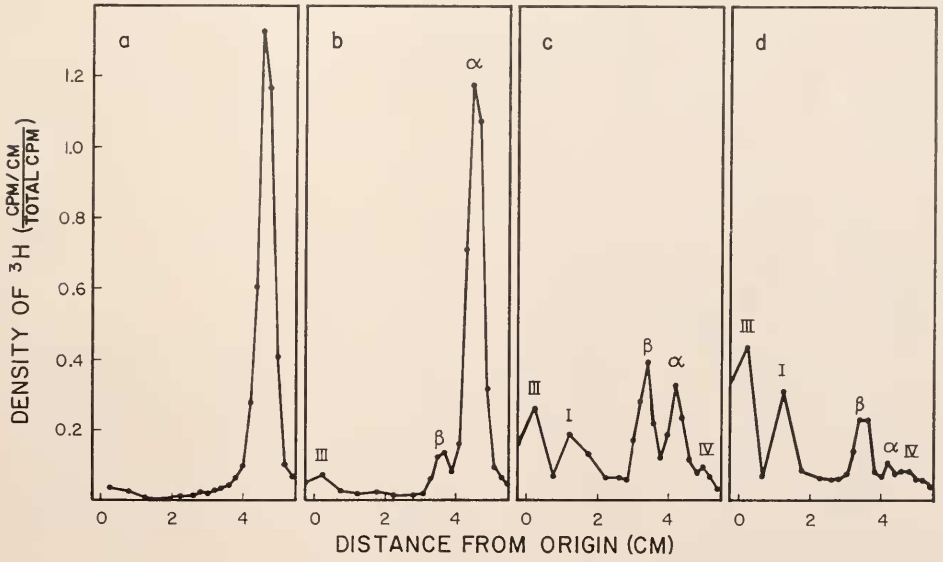

FIGURE 4. Patterns of radioactivity in thin-layer chromatograms of blood extracts, after injection of α-ecdysone-^{3}H. (a) Control, to which the α-ecdysone-^{3}H was added immediately before extraction. (b), (c), (d) Blood from a single pupa, taken 2 hr, 7 hr, and 11 hr, respectively, after injection of 1.2 μg of α-ecdysone-^{3}H. Solvent front was at 18 cm; there was no significant radioactivity in the portion of the chromatograms not shown.

Thin layer chromatography of the extracts distinguished five metabolites of α-ecdysone. Figure 4 shows the dramatic changes in the pattern of metabolites with time, as seen in a pupa injected with 1.2 μg of α-ecdysone-^{3}H. The metabolite labeled β migrates with pure β-ecdysone in this solvent system as well as in cyclohexane-butanol (6:4). The other metabolites resolved in these chromatograms are designated I, III, and IV. Metabolite IV is found in trace quantities in most extracts, but is not seen in control extracts such as that in Figure 4a. Metabolite I does not appear after injection of 8.6 μg of α-ecdysone; instead, another metabolite of slightly different mobility, labeled II, is seen. The mobilities of all these metabolites, as well as those of a number of ecdysone analogs, are shown in Table I.

The quantity of each component was determined from graphs such as those in Figure 4. The minor component IV never represented a fraction high enough to be estimated accurately. Figure 5 shows the time course of appearance of the various metabolites after injection of 1.2 or 8.6 μg of α-ecdysone-^{3}H. There is an initial lag time; all of the α-ecdysone curves extrapolate to 100% at 1–2 hours after

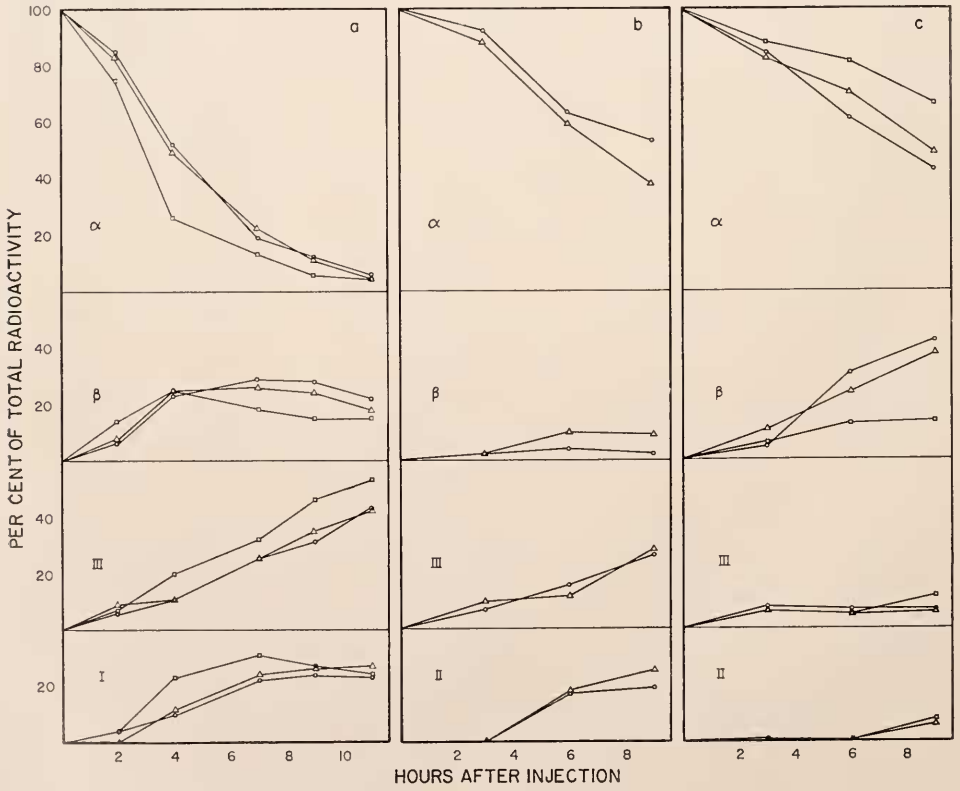

FIGURE 5. Per cent of radioactivity in α-ecdysone and its major metabolites after injection of 1.2 μg of α-ecdysone-^{3}H (a), 8.6 μg of α-ecdysone-^{3}H (b), or 1.2 μg of α-ecdysone-^{3}H and 100 μg of cold β-ecdysone (c) per pupa. II did not appear in series (a); I did not appear in (b) and (c) (see Discussion). In each experiment, the individual pupae are distinguished by different symbols.

TABLE I

Migration in thin-layer chromatography of α-ecdysone, its metabolites, and some ecdysone analogs of known structure

Compound	Structure	R_f / R_f of α-ecdysone (range in parentheses)
α-ecdysone	5β-cholest-7-en-6-one, 2β, 3β, 14α, 22, 25-pentahydroxy (22R) (Huber and Hoppe, 1965)	1.00
β-ecdysone	5β-cholest-7-en-6-one, 2β, 3β, 14α, 20, 22, 25-hexahydroxy (20R, 22R) (Hoffmeister, Gruetzmacher, and Duennebeil, 1967)	0.76 (0.69–0.84)
Metabolite I		0.30 (0.26–0.36)
Metabolite II		0.45 (0.41–0.50)
Metabolite III		0.05 (0–0.06)
Metabolite IV		1.18 (1.04–1.24)
25-deoxyecdysone	5β-cholest-7-en-6-one, 2β, 3β, 14α, 22-tetrahydroxy (22R)	1.50
Rubrosterone	5β-androst-7-en-6, 17-dione, 2β, 3β, 14α-triol (Takemoto, Hikino, Hikino, Ogawa, and Nishimoto, 1968)	1.30
Ponasterone A	5β-cholest-7-en-6-one, 2β, 3β, 14α, 20, 22-pentahydroxy (20R, 22R) (Huppi and Siddall, 1968)	1.45
Ponasterone C	5β-cholest-7-en-6-one, 2α, 3α, 14α, 20, 22, 24-pentahydroxy (Nakanishi, Koreeda, Chang, and Hsu, 1968)	1.35

injection, the time required for distribution of the blood. Excluding the lag time, the decay of α-ecdysone after injection of 1.2 μg is exponential; it is more nearly linear after injection of 8.6 μg. Figure 6 shows the dependence of the rate of breakdown of α-ecdysone upon the concentration of injected α-ecdysone.

When 1.2 μg of α-ecdysone-^{3}H and 100 μg of cold β-ecdysone were injected simultaneously, radioactivity accumulated in β-ecdysone, while II and III were scarcely detectable (Fig. 5c). The loss of counts from α-ecdysone was markedly slower than when the same dose of α-ecdysone was injected alone.

DISCUSSION

Inulin as a label for blood

There is abundant evidence that inulin is excluded from vertebrate cells (Davson, 1964). There are several reasons to believe that this is also true of cells in polyphemus. The inulin volume is the same as the pupal blood volume, estimated by exsanguination. The distribution volume is constant for at least 24 hours; hence inulin is not slowly absorbed or converted to a form that can penetrate cells. After injection of inulin-^{3}H, careful washing of the epidermis removes nearly all radioactivity. The confinement of inulin to the extracellular space makes this compound a very useful tool, both for following the movement of blood and for measuring blood contamination of tissue samples. Levenbook (1958) has already reported the use of ^{14}C-carboxy-inulin for the latter function.

The rates of distribution of inulin and water

It is no surprise that the blood of insects mixes slowly. Craig and Olson (1951) reported, for example, that 20–30 minutes are required for complete mixing to occur

in the squashbug *Anasa tristis* and the cabbage bug *Murgantia histrionica*. It is not unreasonable, then, that two hours should be required in the much larger, diapausing polyphemus pupa. What was surprising was the capricious quality of the insect circulatory system as illustrated by the diverse patterns of mixing in Figure 1: at one point in Figure 1b, there is a fourteen-fold difference in inulin concentration between wing and antenna. The path of injected material, and presumably of an endogenous secretion as well, is not predictable. If hormone binding and breakdown are spatially separated, even a very high binding constant might not ensure efficient utilization of a hormone.

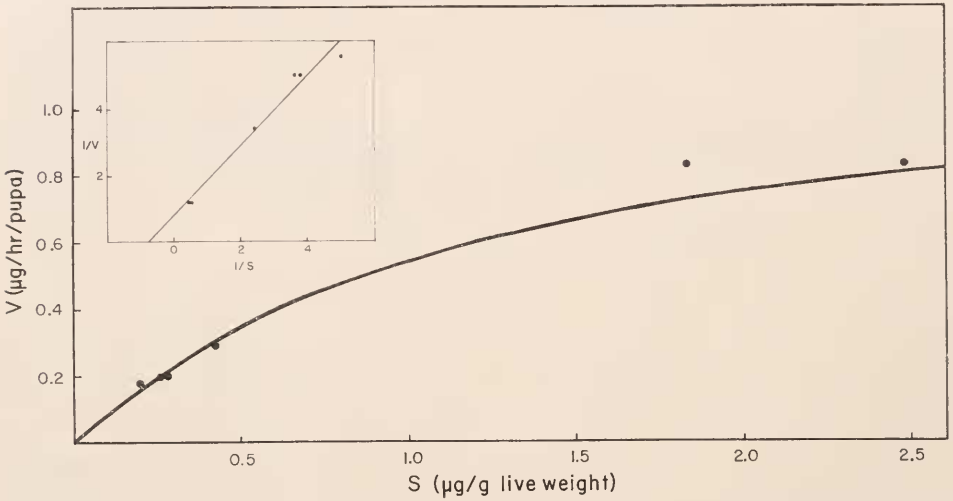

FIGURE 6. The initial rate of metabolism of α-ecdysone as a function of injected dose. The rate was calculated from the first two experimental values in graphs such as those in Figure 5; thus, the lag time was excluded from the calculation. The abscissa (S) is the injected dose divided by the pupa's weight. The curve is plotted from the least-squares best line in the reciprocal plot in the inset.

The rate of distribution of water was not measurably different from that of inulin. From this observation, the *minimum* rate of exchange of water between blood and cells can be calculated to be three-quarters of the amount of water in the blood per hour.

The distribution of ecdysone

Ecdysone reaches its "equilibrium" concentration within about 2 hours after injection. If a carrier is involved, either in the blood or in the target cells, it should be possible to saturate it, making it the rate-limiting step in the hormone's equilibration. The fact that doses up to 10 μg of α-ecdysone or 100 μg of β-ecdysone did not reduce the rate of distribution suggests that a carrier is not involved. In preliminary experiments, we have attempted to detect macromolecules in the plasma capable of binding α-ecdysone. These experiments, using the gel-filtration technique of Hummel and Dreyer (1962), have shown that if carrier macromolecules are present in the plasma of diapausing pupae, they are incapable of

binding more than 10% of the α-ecdysone even when the latter is present at the extremely low concentration of 2×10^{-4} μg/ml.

At "equilibrium," about half of the injected ecdysone has disappeared from the blood. The precise value varies slightly with dose and time, but for a given dose of ecdysone, the level of radioactivity in the blood is fairly constant for at least 36 hours. The fact that the amount of radioactivity in the tissues does not change markedly as the ecdysone is metabolized implies that the bulk of the material in both cells and blood is in solution rather than bound to a receptor. Since the ecdysone volume is 20% larger than the water volume, either 20% of the ecdysone is bound or dissolved in a non-aqueous phase, or ecdysone and its metabolites are slightly concentrated by an active transport mechanism; we cannot distinguish among these possibilities.

The experiments with Malpighian tubules and fat body, as well as the detailed experiments with antennae, gave results consistent with the idea that ecdysone is distributed evenly through the tissues and not accumulated by any one tissue. If all tissues are like the antenna in this respect, they contain ecdysone at approximately its concentration in the blood, and the hormone cannot be removed by a washing procedure which removes essentially all the contaminating blood. The fraction of the injected ecdysone associated with tissues in this way does not increase even when the injected dose is only 0.01 molting unit.

Preliminary experiments indicate that the situation just described prevails in antennal tissue during the 48 hours following injection. Superimposed on this baseline are short periods during which the tissue contains up to 20 times as much radioactivity. One such period occurs 5–6 hours after the injection, and another occurs about 18 hours later. If one extracts antennae containing the baseline amount of radioactivity, α-ecdysone and its metabolites are recovered in proportion to their concentrations in the blood. Details of these experiments will be published later.

If all tissues undergo the periods of extensive binding exemplified by the antenna, these periods must be asynchronous, since synchronous binding of such magnitude would remove all of the ecdysone from the blood. Since there is progressive degradation of active hormone, the amount of ecdysone that must be injected to induce adult development must be determined not only by the number of receptors, but also by the necessity to maintain sufficient active hormone during later periods of binding. Detailed analysis of this situation will become possible only when we know whether it is α-ecdysone or one of its metabolites which is active.

Isolated receptor molecules generally exhibit high affinities for hormones; one expects that this will be true of ecdysone receptors. In contrast, the relation between intact tissues and ecdysone can be described formally as weak affinity.

The metabolism of α-ecdysone

King and Siddall (1969) have provided convincing evidence that insects can convert α-ecdysone to β-ecdysone. We have confirmed this observation in polyphemus pupae; the radioactive metabolite labeled β in our chromatograms not only migrated with authentic β-ecdysone in two different solvent systems, but also accumulated when authentic β-ecdysone was injected.

It seems clear that β-ecdysone is the first metabolite formed from α-ecdysone. Not only is this consistent with the structures of the two compounds and with the kinetic data in Figures 5a and 5b, but it is demonstrated in no uncertain terms by the experiment of Figure 5c. Here β-ecdysone accumulates while the production of other metabolites is retarded. Apparently, at least in polyphemus pupae, the enzyme responsible for the conversion of α to β approaches saturation in the range of α-ecdysone concentrations necessary to provoke adult development (Fig. 6). It is also apparent from a comparison of Figures 5a and 5c that β-ecdysone or one of its metabolites inhibits the conversion of α-ecdysone to β-ecdysone, or else β-ecdysone is converted to α-ecdysone at a significant rate.

The chromatographic mobilities of the metabolites are consistent with their being formed by progressive hydroxylation of α-ecdysone. Ohtaki, Milkman and Williams (1968) have shown that oxygen is necessary for the inactivation *in vivo* of α-ecdysone.

We observe two separate metabolites with mobilities 0.3 to 0.5 times that of α-ecdysone. In the presence of large quantities of α- and β-ecdysone we see metabolite II rather than metabolite I (Fig. 5). A variety of hypothetical pathways are compatible with this kinetic data: I as a precursor of II; II as a precursor of I; or both I and II produced from β along separate pathways.

Thompson, Kaplanis, Robbins and Yamamoto (1967) reported the isolation from the tobacco hornworm of two ecdysone-like materials with mobilities less than β-ecdysone in a chromatographic system similar to ours. One compound was 20,26-dihydroxy-α-ecdysone—a biologically active substance which they suggested might be a product of β-ecdysone. Both its structure and its chromatographic properties indicate that it may be I or II.

Metabolite IV differs from the other radioactive metabolites in being somewhat less polar than α-ecdysone. It appears quite early and never amounts to more than 2% of the radioactivity. For the time being, we defer any further consideration of its significance.

Metabolite III is so immobile in our chromatographic system that it is impossible to be certain that it represents only one compound. The progressive accumulation of this metabolite(s) suggests that it may be at the end of a degradative pathway for α-ecdysone. Since the injected α-ecdysone was labeled on carbons 23 and 24 of the side-chain, the possibility should not be overlooked that III is what remains of the side-chain after oxidative cleavage of a previous metabolite. This kind of degradative pathway has already been suggested by Horn *et al.* (1966).

Previous studies using biological assays (Ohtaki *et al.*, 1968; Ohtaki and Williams, 1970; Karlson and Bode, 1969) have demonstrated exponential inactivation of injected α-ecdysone in dipteran larvae and pupae of the silkworm *Samia cynthia*. Until we know which of the radioactive metabolites have biological activity, a detailed comparison of these studies with ours is not possible.

We gratefully acknowledge the advice of Professor C. M. Williams and his critical reading of the manuscript. Radioactive α-ecdysone was essential to this study and we are indebted to Dr. John Siddall for making it available to Professor Williams' laboratory. We are also grateful to Professor Fotis C.

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After the preparation of this paper, an independent study of ecdysone metabolism came to our attention. Professor K. Nakanishi generously allowed us to read a manuscript describing the results of this study (Moriyama, King, Nakanishi, Okauchi, Siddall and Hafferl, 1970). These authors describe four polar metabolites of α-ecdysone. The first to appear is β-ecdysone; the other three, which they call A, B, and C, correspond to our III, I and II, respectively. A less polar peak, D, corresponding to IV, can also be seen in their chromatograms. They discuss possible structures for some of these metabolites. The results of the two studies are entirely consistent, differing only in quantitative details.

Summary

1. The distribution of inulin, water, and α-ecdysone was studied in pupae of the silkworm *Antheraea polyphemus*. All three compounds reach equilibrium within two hours after injection.

2. Inulin is restricted to the extracellular space, which amounts to 0.49 ml per g live weight.

3. The ecdysone space is slightly larger than the water space. About 50% of the radioactivity derived from α-ecdysone remains in the blood for at least 36 hours.

4. The other 50% is associated with cells in a form which cannot be removed by extensive washing of the intact tissues with saline.

5. Transport of ecdysone to the tissues does not involve a rate-limiting carrier system that can be saturated by 10 μg α-ecdysone or 100 μg β-ecdysone.

6. There is no evidence that ecdysone is accumulated by any one tissue or excretory organ.

7. The metabolism of α-ecdysone was studied by thin layer chromatography of extracts of blood after injection of radioactive α-ecdysone. α-ecdysone is rapidly converted to β-ecdysone and the latter to several more polar metabolites.

8. β-ecdysone either inhibits the conversion of α-ecdysone to β-ecdysone or is itself converted to α-ecdysone at a significant rate.

LITERATURE CITED

Craig, R., and N. A. Olson, 1951. Rate of circulation of body fluid. *Science*, **113**: 648–650.

Davson, H., 1964. *A Textbook of General Physiology*. [Third edition] Little, Brown, Boston, 1166 pp.

Gorski, J., D. Toft, G. Shyamala, D. Smith, and A. Notides, 1968. Hormone receptors: studies on the interaction of estrogen with the uterus. *Recent Progr. Hormone Res.*, **24**: 45–80.

Hoffmeister, H., H. F. Gruetzmacher and K. Duennebeil, 1967. Untersuchungen über die Struktur und biochemische Wirkung von Ecdysteron. *Z. Naturforsch.*, **22b**: 66–70.

Horn, D. H. S., E. J. Middleton and J. A. Wunderlich, 1966. Identity of the moulting hormones of insects and crustaceans. *Chemical Communications*, **1966**: 339–341.

Huber, R., and W. Hoppe, 1965. Die Kristall- und Molekülstrukturanalyse des Insekten-verpupungshormons Ecdyson mit der automatisierten faltmolekülmethode. *Chem. Ber.*, **98**: 2403–2424.

Hummel, V. P., and W. J. Dreyer, 1962. Measurement of protein-binding phenomena by gel filtration. *Biochim. Biophys. Acta*, **63**: 530–532.

Huppi, G., and J. B. Siddall, 1968. Steroids. CCCXXVI. Synthetic studies on insect hormones. 6. The synthesis of ponasterone A and its stereochemical identity with crustecdysone. *Tetrahedron Lett.*, **24**: 1113–1114.

Jensen, E. V., and H. I. Jacobson, 1962. Basic guides to the mechanism of estrogen action. *Recent Progr. Hormone Res.*, **18**: 387–414.

Karlson, P., and C. Bode, 1969. Die Inaktivierung des Ecdysons bei der Schmeissfliege *Calliphora erythrocephala* Meigen. *J. Insect Physiol.*, **15**: 111–118.

Karlson, P., C. E. Sekeris and R. Maurer, 1964. Verteilung von Tritium-markiertem Ecdyson in Larven von *Calliphora erythrocephala*. *Hoppe-Seylers Z. Physiol. Chem.*, **336**: 100–106.

King, D. S., and J. B. Siddall, 1969. Conversion of α-ecdysone to β-ecdysone by crustaceans and insects. *Nature*, **221**: 955–956.

Levenbook, L., 1958. Intracellular water of larval tissues of the southern armyworm as determined by the use of C^{14}-carboxy-inulin. *J. Cell. Comp. Physiol.*, **52**: 329–339.

Moriyama, H., D. S. King, K. Nakanishi, T. Okauchi, J. B. Siddall, and W. Hafferl, 1970. On the origin and metabolic fate of α-ecdysone in insects. *Gen. Comp. Endocrinol.*, in press.

Nakanishi, K., M. Koreeda, M. L. Chang and H. Y. Hsu, 1968. Insect hormones. V. The structures of ponasterones B and C. *Tetrahedron Lett.*, **24**: 1105–1110.

Ohtaki, T., and C. M. Williams, 1970. Inactivation of α-ecdysone and cyasterone by larvae of the fleshfly, *Sarcophaga peregrina*, and pupae of the silkworm, *Samia cynthia*. *Biol. Bull.*, **138**: in press.

Ohtaki, T., R. D. Milkman and C. M. Williams, 1968. Dynamics of ecdysone secretion and action in the fleshfly *Sarcophaga peregrina*. *Biol. Bull.*, **135**: 322–334.

Takemoto, T., Y. Hikina, H. Hikino, W. Ogawa and N. Nishimoto, 1968. Structure of rubrosterone, a novel C_{19} metabolite of insect-moulting substances from *Achranthes rubrofusca*. *Tetrahedron Lett.*, **24**: 3053–3056.

Thompson, M. J., J. N. Kaplanis, W. E. Robbins and R. T. Yamamoto, 1967. 20,26-dihydroxyecdysone, a new steroid with moulting hormone activity from the tobacco hornworm, *Manduca sexta* (Johannson). *Chemical Communications*, **1967**: 650–653.

Weevers, R. DeG., 1966. A lepidopteran saline: effects of inorganic cation concentrations on sensory, reflex and motor responses in a herbivorous insect. *J. Expl. Biol.*, **44**: 163–175.

Williams, C. M., 1970. Hormonal interactions between plants and insects. *In:* E. Sondheimer and J. Simeone, Eds., *Chemical Ecology*. Academic Press, New York.

Wyatt, G. R., 1968. Biochemistry of insect metamorphosis. *In:* W. Etkin and L. Gilbert, Eds., *Metamorphosis: A Problem in Developmental Biology*. Appleton-Century-Crofts, New York.